



Attachment A

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Amendment to the Specification
Clean Version

Please amend the specification as follows:

- *On page 34, lines 5 - 11, replace the paragraph in the specification with the following:*

In the cases of nuclear and mitochondrial localization, short peptide sequences known in the literature can be added to direct localization. An example is the nuclear localization sequence from the SV40 virus T antigen, P-K-K-K-R-K-V (SEQ ID NO.1). For location to membranes, a sequence that directs myristoylation or palmitoylation can be added, usually to one or other end of the protein. Such lipid targeting motifs are reviewed in Ann. Rev Biochem (1988) 57, 69. An amino acid sequence of interest includes the sequence M-G-S-S-K-S-K-P-K-D-P-S-Q-R (SEQ ID NO.2) added to the N-terminus. Location in the cytoplasm is believed to be a default state in the absence of any other localization signals.

- *On page 45, lines 29 - 35, replace the paragraph in the specification with the following:*

α2
 pCGNN was modified by site directed mutagenesis with oligonucleotides VR65, VR119, and VR120 to create pC₄EN. The resulting plasmid has unique restriction sites upstream of the CMV enhancer/promoter region (MluI) and between the promoter and protein coding region (EcoRI).

VR65: TCCCGCACCTTCCGGCCAGCGaaTTccAGAAGCGCGTAT (SEQ ID NO.3)

VR119: GACTCACTATAGGaCGcgTTCGAGCTGCC (SEQ ID NO.4)

VR120: CATCATTGGCAAAGgATTCACTCCTCAGG (SEQ ID NO.5)

- On page 46, lines 1 - 38, replace the page in the specification with the following; page

B. F(36M) domain

F(36M), in which the phenylalanine at amino acid 36 was changed to methionine, was created by mutagenizing a single FKBP domain, cloned into pCGNN with upstream XbaI and downstream SpeI and BamHI sites (Rivera et al., Nat. Med 2:1028-1032, 1996) with oligo VR1 to create pCGNN-F(36M). Two, 3, 4 and 6 tandem copies of F(36M) were created by the stepwise insertion of XbaI-BamHI fragments into SpeI-BamHI-opened vectors.

VR1: GATGGAAAGAAAatgGATTCCCTCCCGG

(SEQ ID NO.6)

C. F(36M) fusion proteins

(a) EGFP fusions

EGFP coding sequence was amplified from pEGFP-1 (Clontech) with oligos VR2 and VR3. The resulting fragment, with upstream XbaI and downstream SpeI sites was inserted into pCGN, a derivative of pCGNN that lacks the SV40 nuclear localization sequence, to create pCGN-EGFP.

VR2: tctagaGTGAGCAAGGGCGAGGAG

(SEQ ID NO.7)

VR3: ggatccttaTTAACTAGTCTTGTACAGCTCGTCCATG

(SEQ ID NO.8)

F(36M)-EGFP fusions were created by inserting XbaI-SpeI fragments containing 3, 4 or 6 copies of F(36M) into the XbaI site of pCGN-EGFP to create pCGN-F(36M)3-EGFP, pCGN-F(36M)4-EGFP, and pCGN-F(36M)6-EGFP .

(b) hGH fusions

An hGH cDNA (506-81) was obtained by RT-PCR amplification of RNA expressed from a cell line containing a genomic hGH gene (Rivera et al., Nat. Med 2:1028-1032, 1996) using oligos VR109 and VR110 to amplify the region from 40 bp upstream of the ATG to 60 bp after the stop codon. The resulting HindIII to EcoRI fragment was cloned into Z₁₂I-PL-2, a derivative of ZHWTx12-IL2-SEAP (Rivera et al., Nat.

a3
On
Med 2:1028-1032, 1996) in which the SEAP gene and SV40 early intron and polyadenylation signal were replaced by a polylinker and the SV40 late polyadenylation signal.

- *On page 47, lines 1 - 37, replace the page in the specification with the following page;*

C4
VR109: aagcttACCACTCAGGGTCCTGTGG

(SEQ ID NO.9)

VR110: gaattcGTGGCAACTTCCA

(SEQ ID NO.10)

To construct hGH fusion proteins, Z_{12} I-hGH-2 was mutagenized with oligos VR185, VR186, and VR187 to create i) an EcoRI site 32 bp upstream of the ATG, ii) an XbaI site immediately after the last amino acid of the signal sequence and iii) a Spe I site immediately after the last amino acid of hGH.

VR185: cacaggaccctGAATTCTaagcttgtggc

(SEQ ID NO.11)

VR186: ATAAGGGAATGGTtctagaGGCACTGCCCT

(SEQ ID NO.12)

VR187: atgccacccgggactagtGAAGCCACAGCTG

(SEQ ID NO.13)

Cloning the resulting EcoRI-SpeI fragment into pC₄EN produced pC₄S₁-hGH which expresses hGH from the CMV enhancer. The XbaI-BamHI fragment of pC₄S₁-hGH was then replaced by XbaI-SpeI fragments containing 2, 3, 4, or 6 copies of F(36M) and a SpeI-BamHI fragment encoding the furin cleavage site-hGH fusion to generate pC₄S₁-F(36M)-FCS-hGH fusions.

A SpeI-BamHI fragment encoding an FCS-hGH fusion protein was generated by amplification of the hGH cDNA with oligos VR4 and VR5.

VR4:actagtGCTAGAAACCGTCAGAAGAGATTCCCAACCATTCCCTTAAGC (SEQ ID NO.14)

VR5: ggatccgggCTAGAAGCCACAGCTGCCCTC (SEQ ID NO.15)

An XbaI-BamHI fragment containing the neo resistance gene downstream of the encephalomyocarditis virus internal ribosome entry sequence (IRES/Neo; Amara et al PNAS 94:10618-23, 1997) was inserted into appropriate SpeI-BamHI-opened vectors to generate pC₄S₁-F(36M)-FCS-hGH/neo and pC₄S₁-hGH/neo vectors.

(c) Insulin fusions

A human insulin cDNA was obtained by RT-PCR amplification of human pancreas polyA+ RNA (Clontech)

using oligos VR220 and VR221 to amplify the region from 9 bp upstream of ATG (EcoRI) to 13 bp after stop codon (BamHI). The resulting EcoRI-BamHI fragment was cloned into pC₄EN to generate pC₄-hIn.

VR220: cGAATTCTtctgccATGGCCCTGTGGATGCGC (SEQ ID NO.16)

VR221: cGGATCCgcaggctgcgtCTAGTTGCAGTAG (SEQ ID NO.17)

- *On page 48, lines 1 - 17, replace the section in the specification with the following;*

A Spel-BamHI fragment encoding an furin cleavage sequence-insulin fusion protein was generated by RT-PCR amplification with oligos VR222 and VR221.

cont

VR222: cACTAGTGCTAGAAACCGTCAGAAGAGATTGTGAACCAACACCTGTGCGGC (SEQ ID NO.18)

VR221: cGGATCCgcaggctgcgtCTAGTTGCAGTAG (SEQ ID NO.19)

The wild type insulin gene and FCS-insulin fusion were mutagenized to i) alter amino acid B10 to Asp, ii) create a FCS at the B-C junction, and iii) create a FCS at the C-A junction, using oligos VR223, VR224, VR225, respectively.

VR223: CCTGTGCGGCTCAgACCTGGTGGAAGC (SEQ ID NO.20)

VR224: CTTCTACACACCCAgGACCaagCGGGAGGCAGAGG (SEQ ID NO.21)

VR225: CCCTGGAGGGGTCCGcGCAGAAGCGTGGC (SEQ ID NO.22)

Mutation of pC₄-hIn produced pC₄-hIn-m3. The mutated FCS-insulin fusions were used to replace the FCS-hGH portion of the pC₄S₁-F(36M)-FCS-hGH fusions to create pC₄S₁-F(36M)-FCS-hIn-m3 fusions.